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Cleft Palate Induced by Mycophenolate Mofetil Is Associated with *miR-4680-3p* and *let-7c-5p* in Human Palate Cells

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Abstract: Background/Objectives: *Cleft palate is a birth defect* associated with environmental and genetic factors. It has been reported that disturbance of microRNAs (miRNAs) and exposure *to medicinal agents during pregnancy can cause cleft palate. Although an association between medicine-induced cleft palate and miRNAs has been suggested, it remains to be fully elucidated. This study aimed to clarify the molecular mechanism underlying mycophenolate mofetil (MPM)-induced inhibition of cell proliferation and miRNA expression in human embryonic palatal mesenchymal (HEPM) cells.* **Methods**: Cell viability, apoptosis, and cell cycle-related markers were evaluated 48 h after MPM treatment. In addition, miRNA levels and expression of their downstream genes were measured, and a rescue experiment was performed using *miR-4680-3p* and/or *let-7c-5p* inhibitors. **Results**: *MPM dose-dependently reduced HEPM cell viability. Additionally, MPM treatment suppressed cyclin-D1, cyclin E1, cyclin-dependent kinase (CDK)-2, and CDK6 expression in HEPM cells. Furthermore,* MPM upregulated *miR-4680-3p* and *let-7c-5p* expression and downregulated the downstream genes of each miRNA. Moreover, *miR-4680-3p* and/or *let-7c-5p* inhibitors alleviated MPM-induced inhibition of cell proliferation. **Conclusions**: These results suggest that MPM-induced cleft palate is associated with *miR-4680-3p* and *let-7c-5p* expression in HEPM cells.

Keywords: mycophenolate mofetil; cleft palate; microRNA ; cell cycle

1. Introduction

Cleft palate (CP) is one of the most common birth defects worldwide, affecting approximately in 1 in every 500 babies in Asia. It has been reported that 70% of cleft lip cases with or without CP (CL/P) are non-syndromic, while the others are syndromic [1]. In humans, the palate forms in two stages, called primary and secondary palates. *Secondary palate formation begins during the 6th week of gestation and is completed by approximately the 10th week. This happens through mesenchymal cell proliferation and epithelial cell fusion, wherein the two pieces of tissue, called palatal shelves, grow downward on either side of the tongue around* the 6th–7th week. Then, around the 7th–8th week, the tongue is retracted between the palatal shelves, which then elevate and fuse above the tongue and primary palate. Finally, the medial epithelial seam breaks down through either apoptosis or migration toward the epithelial triangles on both the oral and nasal sides, or by epithelial-mesenchymal transition until 10th week. Any delay or alteration in apoptosis and/or cell cycle arrest during 6–10 weeks of gestation can result in CP [1].

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The etiology of CP is associated with both genetic and environmental factors [2,3]. Several types of disruptions in the signaling pathway induced by the deletion of genes such those encoding as Sonic Hedgehog protein, WNT, bone morphogenetic protein, fibroblast growth factor, and transforming growth factor result in CP through the inhibition of cell viability [4,5]. Many genes are known to affect proliferation of palatal shelves or bind the each palatal shelf. *Li et al reported that 131 genes involved with CP obtained from systematic review [6]. Environmental factors, such as taking medicine, taking alcohol, and smoking, are suggested to increase the risk for CP through the inhibition of crucial genes or signaling pathways [7]. A recent investigation demonstrated that new environmental factors such as PM2.5 are involved with CP have been reported [8,9]. However, the mechanisms that induce CP are not fully understood.*

MicroRNAs (miRNAs) are single strand RNAs that negatively modulate gene expression by combining to 3'-untranslated regions, resulting in the inhibition of the protein translation or degradation of mRNA transcripts [10,11]. *miRNAs play a vital effect in the formation of palatal shelves* [12–14]. Recent studies have shown that specific miRNAs are associated with the mesenchymal palatal cell proliferation by modulating its downstream genes [15,16]. Moreover, our group and other researchers have reported that medicines such as *all-trans* retinoic acid, dexamethasone, phenytoin, and phenobarbital upregulated specific miRNAs and downregulated its downstream genes *in human embryonic palatal mesenchymal (HEPM) cells* and other related cell types [16–19]. However, as the literature focusing on miRNAs and medicine-induced CP is limited, other medicines may modulate miRNAs against medicine-induced CP.

Mycophenolate mofetil (MPM) is a mycophenolic acid prodrug that has been approved as an immunosuppressive agent. MPM has been prescribed as an alternative medicine for many autoimmune diseases such as nephrotic syndrome and lupus nephritis [20]. Although MPM shows potent immunosuppressive efficacy and fewer side effects than previously developed anti-proliferative immunosuppressive agents [21], *several studies have reported that maternal exposure to MPM causes embryo teratogenicity, including CL/P and microtia [22,23].* Using the GeneCard database, Lin *et al* reported that several molecules (MDM2, RPL5, and TP53) may be involved in MPM-induced CL/P [24]. However, the involvement of miRNA regulation in MPM-induced CP remains unknown. In the present study, we investigated the involvement of miRNAs in MPM-induced inhibition of cell proliferation using HEPM cells.

2. Materials and Methods

2.1. Cell Culture

HEPM cells were purchased from American Type Culture Collection (CRL-1486, Manassas, VA, USA) and kept in Minimum Essential Medium Eagle-alpha modification medium (αMEM; Fujifilm-Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (Millipore-Sigma, St Louis, MO, USA), penicillin (10 U/mL), and streptomycin (10 μg/mL; Fujifilm-Wako Pure Chemical Corporation). The cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Cell Viability Assay

HEPM cells were seeded in 5000 cells per 96 well plate (n=6) and treated with several concentrations (0–10 μM) of MPM (Tokyo Kasei Co. Ltd., Tokyo, Japan) after 24 h of cell seeding. After 48 h of treatment, the cell viability was measured using Alamar Blue (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Apoptosis Assay

HEPM cells were seeded in 10000 cells per 8-well chamber slides (Biomedical Sciences Inc., Tokyo, Japan) and treated with 1 μM MPM or vehicle after 24 h seeding. After 48 h of treatment,

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apoptosis-positive cells were calculated using ApoTracker Green (BioLegend, San Diego, CA, USA) according to our previous litearture [19]. Copper dichloride was used as a positive control [25] and Hoechst 33342 (Nacalai Tesque, Kyoto, Japan) was used as a nuclear counterstaining.

2.4. Western Blotting

HEPM cells were seeded in 200000 cells per 35 mm dish and treated with 1 μM MPM or vehicle after 24 h seeding. After 48 h of treatment, we washed PBS twice. To collect the protein, we added 100 μL ice-cold RIPA buffer (Nacalai Tesque) containing a protease inhibitor (Nacalai Tesque) and waited on ice. After 5 min, we scraped the cells and sonicated (15%, 5 sec, 3 times, Branson, Danbury, CR, USA). We subsequently centrifuged (20,000 × *g* for 20 min at 4°C) and collected supernatant as protein. The protein concentration was calculated as previously described [25–27]. Protein samples (10 μg) were applied to gradient (5-20 %) precast sodium dodecyl sulfate-polyacrylamide gel (ATTO, Tokyo, Japan) and transferred onto polyvinylidene difluoride (PVDF) membranes using Trans Turbo Blot (Bio-Rad Laboratories). The antibodies used are listed in Table 1. Band intensity was measured using Image J software (NIH, Bethesda, MD)

Table 1. Antibody lists for western blotting.

Antibody name	Vendor	Catalog number	Concentration
-ACTIN	Medical & Biological Laboratories	M177-3	1:3000
BAX	Santa Cruz Biotechnology	sc-20067	1:1000
Cleaved CASPASE-3	Cell Signaling Technology	9661	1:3000
CCND1	Santa Cruz Biotechnology	sc-8396	1:500
CCNE	Santa Cruz Biotechnology	sc-377100	1:1000
CDK2	Santa Cruz Biotechnology	sc-6248	1:1000
CDK4	Santa Cruz Biotechnology	sc-56277	1:1000
CDK6	Santa Cruz Biotechnology	sc-53638	1:500
BACH1	Santa Cruz Biotechnology	sc-271211	1:500
PAX3	Santa Cruz Biotechnology	sc-376204	1:500
ERBB2	Santa Cruz Biotechnology	sc-393712	1:1000
JADE1	Proteintech Japan	28472-1-AP	1:2000
Rabbit IgG HRP	Cell Signaling Technology	7074	1:10000
Mouse IgG HRP	Cell Signaling Technology	7976	1:10000

2.5. Bromodeoxyuridine (BrdU) Incorporation Assay

HEPM cells were seeded in 10000 cells per 8-well chamber slides (Biomedical Sciences Inc.) and treated with 1 μM MPM or vehicle (0.1% DMSO). After 48 h of treatment, the cells were incubated with BrdU (100 μg/mL, Sigma-Millipore) as we previously described [19].

2.6. Quantitative RT-PCR

HEPM cells were seeded in 200000 cells per 35 mm dish and treated with 1 μM MPM or vehicle after 24 h seeding. After 48 h of treatment, we washed PBS twice. To collect the total RNA, we extracted RNA from HEPM cells using a QIAshredder and miRNeasy Mini Kit (QIAGEN, Valencia, CA, USA) (n=3–5) [28,29]. miRNA expression was tested according to our previous investigation [30]. The expression level of target miRNAs was standardized to U6 expression levels.

2.7. Rescue Experiments Using miRNA Inhibitor

To rescue the effect of MPM, we used miRNA inhibitor was treated. We obtained miRNAs from BIONEER Ltd or Integrated DNA Technologies (IDT), respectively. HEPM cells were seeded in 5000 cells per 96 well plate (n=6). After 6 h of cell seeding, the HEPM cells were treated with *hsa-7c-5p*

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inhibitor (3 pmol; BIONEER Ltd., Daejeon, Korea), control miR inhibitor (3 pmol; BIONEER Ltd.), *hsa-iR-4680-3p* inhibitor (3 pmol: IDT, Coralville, IA, USA), or control miR inhibitor (3 pmol: IDT), using FuGENE SI Transfection Reagent (Promega, Madison, WI, USA), according to the manufacturer's protocol. Cells were treated with 1 μM MPM 24 h after transfection. After 48 h of treatment, cell viability was evaluated using Alamar Blue.

2.8. Statistical Analysis

Comparisons between two or more groups were conducted using Student's t-test or Tukey's test, respectively. All statistical analyses were conducted using IBM SPSS Statistics 26.0 for Windows (IBM Corp., Armonk, NY, USA). Values of $P<0.05$ were regarded statistically significant.

3. Results

3.1. MPM Inhibits Cell Proliferation via G1 Arrest in HEPM Cells

First, we performed a cell viability assay to determine whether an MPM-induced reduction in cell viability in HEPM cells. MPM treatment inhibited the viability of HEPM cells in a dose-dependent manner (Figure 1). We selected 1 μM for the following experiments since the suppression effect plateaued at this dose.

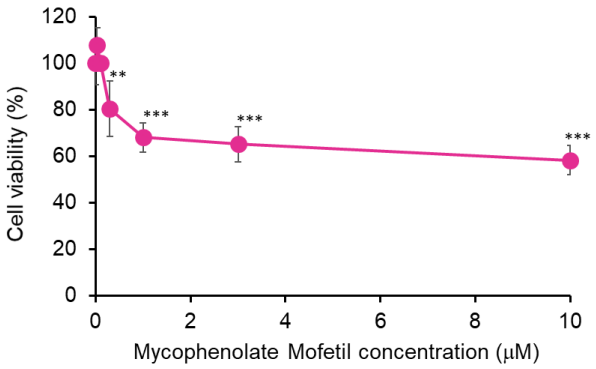

Figure 1. MPM inhibited cell proliferation in HEPM cells.

Proliferation of HEPM cells treated with MPM (0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μM) for 48 h. Data are presented as the mean ± standard deviation (SD). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus control (n=6).

Several studies have reported that medicine-induced inhibition of mesenchymal palatal cell proliferation induces apoptosis and cell cycle arrest [14,31,32]. Therefore, we tested the effect of apoptosis and cell cycle arrest in MPM-treated HEPM cells. As shown in Figure 2A, treatment with MPM did not increase apoptosis-positive cells monitored by Apotracher, whereas copper dichloride (positive control) treatment activated apoptosis-positive cells. To support the result of Apotracker, we demonstrated that the protein expression of cleaved caspase-3, an indicator of apoptosis, was not increased by MPM treatment (Figure 2B).

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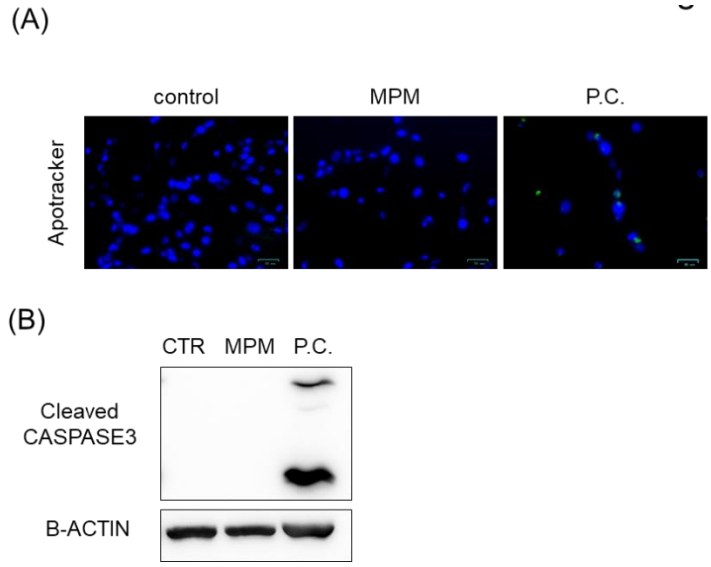

Figure 2. MPM-induced cell proliferation is not associated with apoptosis in HEPM cells. (A)Apotracker staining of HEPM cells after treatment with 1 μM MPM for 48 h. The nuclei were counterstained with Hoechst 33342. Copper dichloride (500 μM 24 h) was used as a positive control. Scale bar, 50 μm. (B) Western blotting of HEPM cells treated with 1 μM MPM for 48 h. β-ACTIN was served as an internal control. Copper dichloride (500 μM M 24 h) was used as the positive control (P.C.).

Next, we monitored the cell cycle progression using a BrdU incorporation assay and found that the number of BrdU-positive cells was significantly reduced by MPM treatment (Figure 3A). To further investigate the molecular mechanisms underlying MPM-induced cell cycle arrest (G1-arrest), we tested cyclins and cyclin-dependent kinases (CDK) and found that MPM treatment reduced CCND1, CCNE, CDK2, and CDK6 levels, whereas those of CDK4 were comparable (Figure 3B). These results indicate that MPM induces cell cycle arrest (G1-arrest) by suppressing CCND1/CDK6 and CCNE/CDK2 in HEPM cells.

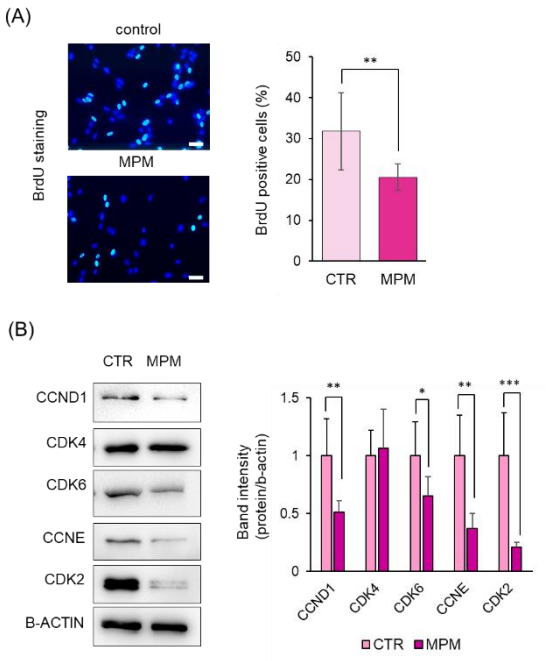

Figure 3. MPM-induced cell proliferation is associated with G1 arrest in HEPM cells. (A) BrdU staining (green) of HEPM cells after treatment with 1 μM MPM for 48 h. The nuclei were counterstained with Hoechst 33342 (blue). Scale bar, 50 μm. Graph shows the quantification of BrdU positive cells. Data are presented as the mean

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± standard deviation (SD). **$p < 0.01$ (n=6). (B) Western blotting of HEPM cells treated with 1 μM MPM for 48 h. β-ACTIN was served as an internal control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (n=3).

3.2. MPM Modulates let-7c-5p/miR-4680-3p and Its Downstream Genes in HEPM Cells.

Recently, miRNAs have been suggested to be associated with the CL/P etiology [12,13]. Suzuki and Li *et al.* identified miRNAs associated with human CP-related genes by performing systematic reviews, bioinformatic reviews, cell proliferation assays, and qPCR [6,33]. Moreover, Fu *et al* identified two miRNAs from a CL/P patient database and *in vitro* experiments [15]. In the present study, we measured the expression of seven miRNAs (*let-7c-5p, miR-133b, miR-140-5p, miR-193a-3p, miR-374a-5p, miR-381-3p,* and *miR-4680-3p*) using qPCR. We found that MPM treatment significantly increased *let-7c-5p* and *miR-4680-3p* expression, whereas the expression of the other five miRNAs was unaltered in HEPM cells (Figure 4A). To further investigate the effects of *let-7c-5p* and *miR-4680-3p* on downstream genes (*BACH1* and *PAX3* for *let-7c-5p* and *ERBB2* and *JADE1* for *miR-4680-3p*) [30,34], we conducted western blot analysis and found that MPM treatment suppressed BACH1, PAX3, ERBB2, and JADE levels (Figure 4B). These findings indicate that MPM modulates *let-7c-5p* and *miR-4680-3p* upregulation and inhibition of their downstream genes.

Figure 4.

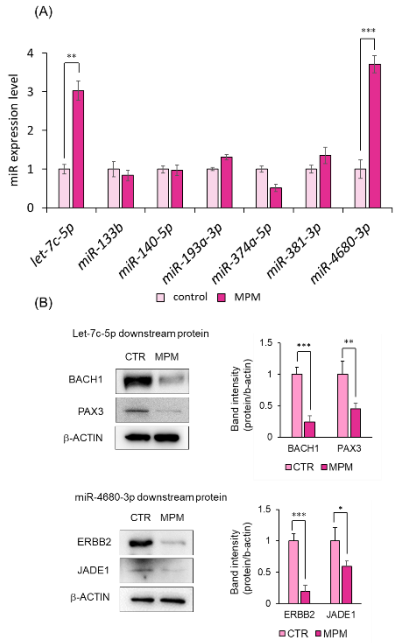

Figure 4. MPM upregulated *let-7c-5p* and *miR-4680-3p* in HEPM cells. (A)Quantitative RT-PCR of *let-7c-5p, miR-133b, miR-140-5p, miR-193a-3p, miR-374a-5p, miR-381-3p,* and *miR-4680-3p* after treatment of HEPM cells with 1 μM MPM for 48 h. Data are presented as the mean ± standard deviation (SD). **$p < 0.01$ and ***$p < 0.001$ (n=3). (B) Western blotting of HEPM cells treated with 1 μM MPM for 48 h. β-ACTIN was served as an internal control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (n=3).

3.3. Inhibition of let-7c-5p and/or miR-4680-3p Alleviated MPM-Induced Cell Proliferation Activity in HEPM *Cells.*

To further investigate the contributions of *let-7c-5p* and *miR-4680-3p*, we transfected HEPM cells with *let-7c-5p* and/or *miR-4680-3p* inhibitors to examine whether *let-7c-5p* and/or *miR-4680-3p* alleviated the inhibition of HEPM cell proliferation following MPM treatment. Transfection with *let-7c-5p* and *miR-4680-3p* inhibitors suppressed expression of their respective targets by more than 80% under our experimental conditions (Figure 5A). Finally, we treated *let-7c-5p* and/or *miR-4680-3p* inhibitor whether MPM-induced cell proliferation inhibition was attenuated or not. We found that the *let-7c-5p* or *miR-4680-3p* inhibition partially alleviated the MPM-induced reduction in cell

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proliferation (Figure 5B). Moreover, treatment with both *let-7c-5p* and *miR-4680-3p* inhibitors fully protected MPM-induced cell proliferation inhibition in HEPM cells (Figure 5B). These results suggest that *let-7c-5p* and *miR-4680-3p* were associated with MPM-induced inhibition of HEPM cell proliferation.

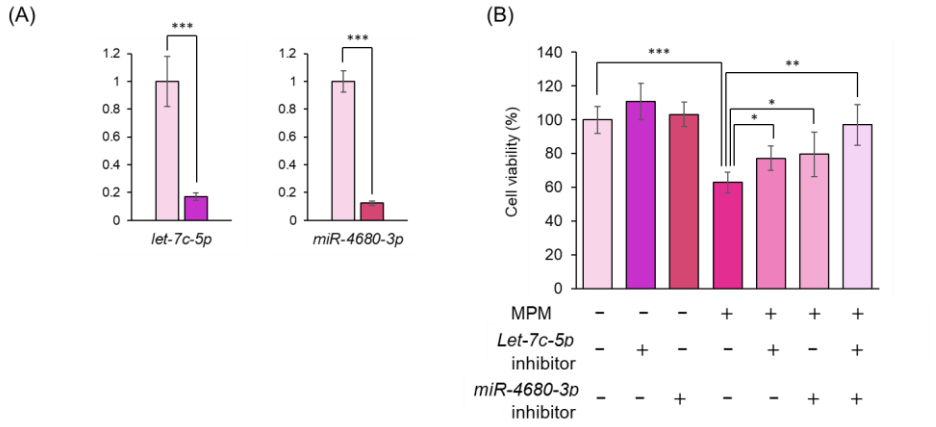

Figure 5. Inhibition of *let-7c-5p* and *miR-4680-3p* alleviated MPM-induced cell proliferation inhibition in HEPM cells. (A)Quantitative RT-PCR analysis of *let-7c-5p* or *miR-4680-3p* expression after transfected HEPM cells with *let-7c-5p* inhibitor or *miR-4680-3p* inhibitor for 24 h. Data are presented as the mean ± standard deviation (SD). ***$p < 0.001$ (n=3). (B) Proliferation of HEPM cells treated with 1 μM MPM and/or *let-7c-5p* inhibitor and/or *miR-4680-3p* inhibitor for 48 h. Data are presented as the mean ± standard deviation (SD). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (n=6).

4. Discussion

In this study, we examined the role of miRNAs in MPM-induced toxicity in HEPM cells. MPM treatment reduced the cell viability in a dose-dependent manner (Fig. 1) and downregulated the expression of CCND1, CCNE, CDK2, and CDK6 in HEPM cells (Fig. 3B). Several reports have suggested that MPM-induced toxicity derives from its inhibition purine synthesis (inosine-5′-monophosphate dehydrogenase) [35,36]; therefore, it is feasible that MPM induces cell cycle arrest (Fig. 3A, 3B). Additionally, MPM treatment induced *let-7c-5p* and *miR-4680-3p* expression and downregulated the expression of their downstream genes (Fig. 4B). Notably, the inhibition of *let-7c-5p* or *miR-4680-3p* partially alleviated MPM-induced cell inhibition and inhibition of both *let-7c-5p* and *miR-4680-3p* fully protected HEPM cells against MPM-induced cell proliferation inhibition (Fig. 5C).

Cyclins and CDKs play crucial roles in the regulation of cell cycle events [37]. When cells in the G0 phase enter the cell cycle, CDK4/CDK6 forms active complexes with CCND and other proteins, such as phosphorylated retinoblastoma protein (pRb), which activates the transition step from G1 to S phase [38]. Moreover, the CDK2 and CCNE complex phosphorylates and inactivates Rb family members [39]. The subsequent release of transcription factors (e.g., E2F) allows the cells to transition from G1 to S phase. Many cancers are known to increase CCND levels, and CCND activates signaling pathways, such as the MAPK kinase and PI3K/Akt pathways [40]. CCNE overexpression accelerates G1 phase progression in cancer patients [41]. CDK2 inhibition reduces the viability of human colon cancer cells [42]. CDK4/6 inhibitors, such as palbociclib, ribociclib, and abemaciclib have been approved for patients with HER2 negative breast cancer [43]. We demonstrated the number of BrdU-incorporated cells were significantly reduced with MPM treatment (Fig. 3A). Since the BrdU incorporation assay is an indicator of S phase, we investigated the molecular mechanism related to G1 phase proteins and found that MPM reduced CCND1, CDK6, CCNE, and CDK2 levels (Fig. 3B). Therefore, it is reasonable to conclude that MPM-induced cell viability reduction is G1-arrest through the suppression of CCND1/CDK6 and CCNE/CDK2 in HEPM cells.

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Several studies have reported that miRNA-gene networks involved with craniofacial development [44,45]. Li *et al.* showed that overexpression and/or knock out mice of *mmu-miR-17-92* cluster leads to CL and CP by modulating the BMP signaling pathway [46]. Polymorphisms in miR-140 and miR-4260 are associated with non-syndromic CL/P in humans [47,48]. Li and Suzuki *et al.* showed that several miRNAs *(hsa-miR-133b, hsa-miR-140-5p, hsa-miR-374a-5p, hsa-miR-381a-3p,* and *hsa-miR-4680-3p)* involved with development of human palate by combinating multi experiments such as systematic reviews, bioinformatics analyses, and cell viability assays [6,33]. Fu *et al.* reported that *hsa-let-7c-5p* and *hsa-miR-193a-3p* involved with CP using the patients with CP and *in vitro* experiment using HEPM cells [15]. We measured the seven (*hsa-let-7c-5p, hsa-miR-133b, hsa-miR-140-5p, hsa-miR-193a-3p, hsa-miR-374a-5p, hsa-miR-381a-3p,* and *hsa-miR-4680-3p*) miRNAs and demonstrated that MPM significantly induced *let-7c-5p* and *miR-4680-3p* expression level in HEPM cells (Fig. 4A). In addition, *let-7c-5p* or *miR-4680-3p* specific inhibitors partially alleviated and combined treatment with *let-7c-5p* and *miR-4680-3p* inhibitors fully protected against MPM-induced suppression of HEPM cell proliferation (Fig. 5B), indicating that *let-7c-5p* and *miR-4680-3p* play an important role in MPM-induced toxicity. Human *let-7c-5p* is located on chromosome-21 and *let-7c-5p* are involved in cell proliferation [49]. Overexpression of *let-7c-5p* suppresses human breast cancer and osteoblasts by downregulating CCDN1 [50,51]. Moreover, we previously reported that *let-7c-5p* and its inhibitor partially attenuated the phenobarbital-induced cell viability reduction by modulating BACH1 and PAX3 in HEPM cells [30]. Human *miR-4680-3p* is located on chromosome-10 and *miR-4680-3p* are expressed in gastric cancer [52]. *miR-4680-3p* and *miR-4680-3p* inhibitors partially attenuated the *all-trans* retinoic acid- and phenytoin-induced inhibition of cell proliferation through the regulation of ERBB2 and JADE1 [19,34]. Since these miRNAs are associated with several genes, they may play an important role in palate development by modulating downstream genes.

miRNAs negatively regulate downstream genes [53]. We previously identified that *let-7c-5p* inhibitor induces two CP-associated genes (*BACH1* and *PAX3*) in humans [30]. As expected, both genes were downregulated following MPM treatment (Fig. 4B). *BACH1* is ubiquitously expressed in mammals and is involved in multiple events, such as cell cycle and proliferation, through the modulation of the Wntfig/β-catenin signaling pathway [54,55]. *PAX3* is essential for neural crest development. Knockout of *PAX3* results in CP via downregulation of the BMP signaling pathway [56], and a *PAX3* variant is associated with non-syndromic CL/P in humans [57]. As for *miR-4680-3p*, *ERBB2* and *JADE1* have been reported as downstream genes in HEPM cells [34]. We found that the expression of both genes was attenuated by MPM treatment in HEPM cells (Figure 4B). ERBB2 (HER2) is a member of the ERBB-receptor tyrosine kinase family, which includes epidermal growth factor receptor (EGFR). ERBB2 is a target in ERBB2 positive breast cancer [58]. ERBB2 is associated with cell proliferation, migration, and differentiation through the modulation of signaling cascades such as the MAPK/ERK and PI3K/AKT/mTOR pathways [59]. ERBB2 downregulation by all-trans retinoic acid or siRNA reduced cell viability via the ERK1/2 signaling pathway in HEPM cells [34]. JADE1 (PHF17) is a transcription factor and its inhibition reduces cell viability in cultured epithelial cell lines and primary fibroblasts [60]. JADE1 is known to regulate the Wnt/β-catenin signaling pathway [61]. JADE1 inhibition by siRNA reduces HEPM cell viability [34]. As these genes are associated with several signaling pathways related to proliferation, these miRNA-mRNA networks may play a crucial role in palate development by modulating these signaling pathways.

5. Conclusions

In conclusion, we demonstrated that MPM inhibits cell proliferation by inducing the expression of *let-7c-5p* and *miR-4680-3p* and downregulating their downstream genes. This is the first report to show the involvement of miRNAs in MPM-induced inhibition of palate cells. Since miRNAs are potential therapeutic agents [62], our present investigation may contribute to clinical protocols against CP development. Although further investigation is needed to understand how *let-7c-5p* and *miR-4680-3p* regulate the G1 phase, our findings may aid in understanding the etiology of CP.

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